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EXAMINER	
PAK, YONG D	

ART UNIT	PAPER NUMBER
1652	

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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/031,695	HAUER ET AL.	
	Examiner	Art Unit	
	Yong D. Pak	1652	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on *11 April 2007*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-12,14 and 16-18* is/are pending in the application.

4a) Of the above claim(s) *1-11* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *12,14 and 16-18* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☒ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

This application is a 371 of PCT/EP00/07252.

The amendment filed on April 11, 2007, amending claims 12, 14 and 17-18, has been entered.

Claims 1-12, 14 and 16-18 are pending. Claims 1-11 are withdrawn. Claims 12, 14 and 16-18 are under consideration.

Response to Arguments

Applicant's amendment and arguments filed on April 11, 2007, have been fully considered and are deemed to be persuasive to overcome some of the rejections previously applied. Rejections and/or objections not reiterated from previous office actions are hereby withdrawn.

Claim Rejections - 35 USC § 112

Claim 12 and claims 14 and 16-18 depending therefrom are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 12 recites the phrase "is derived from *Bacillus megaterium* cytochrome P450 monooxygnease with an amino acid sequence comprising SEQ ID NO:". The metes and bounds of this phrase are not clear to the Examiner. Literally, while the term

"derived" means "to isolate from or obtain from a source", the above term could also mean "to arrive at by reasoning i.e., to deduce or infer" or also as "to produce or obtain from another substance". Therefore, it is not clear to the Examiner either from the specification or from the claims as to what applicants mean by the above phrase. It is not clear to the Examiner whether the mutants of the monooxygnease "derived from *Bacillus megaterium* cytochrome P450 monooxygnease with an amino acid sequence comprising SEQ ID NO:2" encompasses mutants of a single specific enzyme (SEQ ID NO:2), or whether it encompasses mutants of any monooxygenase, including (1) monooxygenase isolated from any other source and (2) any or all variants, mutants and recombinants of SEQ ID NO:2; and labeled as a monooxygenase "derived from *Bacillus megaterium* cytochrome P450 monooxygnease with an amino acid sequence comprising SEQ ID NO:2". As applicants have not provided a definition for the above phrase, Examiner has interpreted the claims broadly to mean that a monooxygenase "derived from *Bacillus megaterium* cytochrome P450 monooxygnease with an amino acid sequence comprising SEQ ID NO:" encompasses polypeptides which are recombinants, variants or mutants of any monooxygenase. Examiner has given the same interpretation while considering the claims for all other rejections.

Applicants argue that the phrase is not intended to refer to the fact that the sequence is limited to being physically isolated from *B. megaterium*, but is "intended to refer to the fact that the sequence is preferably based on the sequence of *B. megaterim*". Examiner respectfully disagrees. The claim does not exclude monooxygenase that is physically isolated from *B. megaterium*. Also, applicant's

argument that the phrase encompasses monooxygenases "based" on the sequence of the monooxygenase of *B. megaterium* (SEQ ID NO:2) does not address the metes and bounds of the phrase, but in a way agrees with Examiner's interpretation that the phrase encompasses any recombinants, variants and mutants of the monooxygenase of SEQ ID NO:2 or any monooxygenase.

Hence the rejection is maintained.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 12, 14 and 16-18 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claims 12, 14 and 16-18 are drawn to a method for the enzymatic production of subterminally hydroxylated aliphatic carboxylic acids with a cytochrome P450 monooxygenase derived from a *Bacillus megaterium* cytochrome P450 monooxygenase BM-3 with an amino acid sequence comprising SEQ ID NO:2 containing a functional mutation at position 87 and 188 and optionally at least one additional functional mutation at positions 26, 47, 72, 74 and 354.

It is noted that MPEP 2111.01 states that "[d]uring examination, the claims must be interpreted as broadly as their terms reasonably allow." In this case, the limitation of "containing" mutations at positions 87 and 188 and optionally at positions 26, 47, 72 and 354 provide no description on the structure of other parts of the mutant monooxygenase because (1) the claimed variant is not limited to only those mutations at positions 87, 188 and 26, 47, 72 and 354, and (2) the transitional term "containing" is inclusive or open-ended and does not exclude additional, unrecited elements, such as mutations at other positions. Therefore, while the mutant monooxygenase comprises the recited mutations/substitutions, the same mutant comprises any amino acids in any other positions. Therefore, Examiner has interpreted the claims broadly to encompass a method of using a polynucleotide encoding mutants of any monooxygenase, including any monooxygenase isolated from any sources and including any or all variants, recombinants, and mutants thereof, comprising one or more amino acid mutation/substitution at amino acid positions corresponding to 87 and 188 and optionally at positions 26, 47, 72 and 354 of SEQ ID NO:2 and one or more amino acid mutation/substitutions at any other amino acid positions. Therefore, the claims encompass a method for the production of subterminally hydroxylated aliphatic carboxylic acids using a polynucleotide encoding mutant monooxygenase having any structure.

The specification only teaches a method for hydroxylating 15-para-nitrophenoxycarboxylic acids (pNCA), 12-pNCA, 10-pNCA or 8-pNCA with a polynucleotide encoding a mutant of a cytochrome P450 monooxygenase of SEQ ID

NO:2, wherein the mutant consists of mutations at position 26, 47, 74, 87, 188 and/or 354 of SEQ ID NO:2. These limited examples are not enough and does not constitute a representative number of species to describe the whole genus and there is no evidence on the record of the relationship between the structure of a modified cytochrome P450 monooxygenase of SEQ ID NO:2 consisting of substitutions at residues 26, 47, 74, 87, 188 and/or 354 of SEQ ID NO:2 and the structure of any recombinants, variants and mutants of any cytochrome P450 monooxygenase derived from SEQ ID NO:2. Therefore, the specification fails to describe a representative species of the genus comprising variants and mutants of any recombinants, variants and mutants of any cytochrome P450 monooxygenase, derived from SEQ ID NO:2 or from any source, used to produce subterminally hydroxylated aliphatic carboxylic acids.

Given this lack of description of the representative species encompassed by the genus of the claims, the specification fails to sufficiently describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize that applicants were in possession of the inventions of claims 12, 14 and 16-18.

Applicant is referred to the revised guidelines concerning compliance with the written description requirement of U.S.C. 112, first paragraph, published in the Official Gazette and also available at www.uspto.gov.

In response to the previous Office Action, applicants have traversed the above rejection.

Applicants argue that the claims are fully described since claim 12 has been amended to recite "a nucleic acid sequence encoding a monooxygenase which is

derived from *Bacilus megaterium* cytochrome P450 monooxygnease BM-3 with an amino acid sequence comprising SEQ ID NO:2 containing a functional mutation at positions 87 and 188 and optionally at positions 26, 47, 72, 74 and 354". Examiner respectfully disagrees. Applicants' argument on page 9 of the Remarks, that the phrase "is derived *from B. megaterium*" is "not intended to refer to the fact that the sequence is limited to being physically isolated from *B. megaterium*", is in a way in agreement with Examiner's interpretation that the phrase encompasses any recombinants, variants and mutants of monooxygenase isolated from any other source and labeled as a monooxygenase "derived from *Bacillus megaterium*" in addition to any recombinants, variants and mutants of the monooxygenase having the amino acid sequence of SEQ ID NO:2. Therefore, the claims are not only drawn to mutants of SEQ ID NO:2 comprising the recited substitutions, but any mutants of any monooxygenase "derived" from SEQ ID NO:2 or "derived" from any monooxygenases isolated from any or all source, including any or all mutants, variants and recombinants thereof, comprising the recited substitutions. The genus comprising any or all recombinants, variants and mutants of any monooxygenase does not possess any common attributes other than having monooxygenase activity. Therefore, the specification lacks description of a representative number of species to describe the whole genus. As discussed in the written description guidelines, the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by

functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. A representative number of species means that the species which are adequately described are representative of the entire genus. **Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus.** Satisfactory disclosure of a representative number depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species cannot be achieved by disclosing only one species within the genus. In the instant case the claimed genus used in the method includes species which are widely variant in structure. As such, the disclosure solely functional features present in all members of the genus is sufficient to be representative of the attributes and features of the entire genus.

Hence the rejection is maintained.

Claims 12, 14 and 16-18 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of enzymatic production of specific subterminally hydroxylated aliphatic carboxylic acids by using a polynucleotide encoding a mutant of a cytochrome P450 monooxygenase of SEQ ID NO:2, wherein the

mutant consists of a substitution at positions 87 and 188 and optionally at positions 26, 47, 72, 74 and 354 and using 15-para-nitrophenoxycarboxylic acids (pNCA), 12-pNCA, 10-pNCA or 8-pNCA as substrates, does not reasonably provide enablement for a method for the production of any subterminally hydroxylated aliphatic carboxylic acids using a polynucleotide encoding a mutant of any cytochrome P450 monooxygenase. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention commensurate in scope with these claims. (See rejection of "derived" under 35 U.S.C. 112, 2nd paragraph).

Factors to be considered in determining whether undue experimentation is required, are summarized in In re Wands (858 F.2d 731, 8 USPQ 2nd 1400 (Fed. Cir. 1988)) as follows: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claim(s).

Claims 12, 14 and 16-18 are drawn to a method for the enzymatic production of subterminally hydroxylated aliphatic carboxylic acids with a cytochrome P450 monooxygenase derived from a *Bacillus megaterium* cytochrome P450 monooxygenase BM-3 with an amino acid sequence comprising SEQ ID NO:2 containing one functional mutation at positions 87 and 188 and optionally at least one additional function mutation in positions 26, 47, 72, 74 and 354.

It is noted that MPEP 2111.01 states that "[d]uring examination, the claims must be interpreted as broadly as their terms reasonably allow." In this case, the limitation of "containing" mutations at positions 87, 188 and 26, 47, 72 and 354 provide no description on the structure of other parts of the mutant monooxygenase because the claimed variant is not limited to only those mutations at positions 87, 188 and 26, 47, 72 and 354, since the transitional term "containing" is inclusive or open-ended and does not exclude additional, unrecited elements. Therefore, while the mutant monooxygenase comprises the recited mutations/substitutions, the same mutant comprises any amino acids in any other positions. Therefore, Examiner has interpreted the claims broadly to encompass a method for the production of any subterminally hydroxylated aliphatic carboxylic acids using a polynucleotide encoding mutants of any monooxygenase isolated from any or all sources, including any or all variants, recombinants, and mutants thereof, comprising one or more amino acid mutation/substitution at amino acid positions corresponding to 87, 188 and 26, 47, 72 and 354 of SEQ ID NO:2 and one or more amino acid mutation/substitution at any other amino acid positions. Therefore, the claims encompass a method for the production of any subterminally hydroxylated aliphatic carboxylic acids using a polynucleotide encoding mutant monooxygenase having any structure.

The scope of the claims is not commensurate with the enablement provided by the disclosure with regard to the extremely large number of P450 monooxygenase variants and mutants, broadly encompassed by the claims. The claims encompass compounds with widely varying structure and properties. However, in this case the

disclosure is limited to a method for hydroxylating 15-para-nitrophenoxycarboxylic acids (pNCA), 12-pNCA, 10-pNCA or 8-pNCA with a polynucleotide encoding a mutant cytochrome P450 monooxygenase of SEQ ID NO:2, wherein the mutant consists of mutations at residue 26, 47, 74, 87, 188 or 354 of SEQ ID NO:2.

Since the amino acid sequence of a protein determines its structural and functional properties, predictability of which changes can be tolerated in a protein's amino acid sequence and obtain the desired activity requires a knowledge of and guidance with regard to which amino acids in the protein's sequence, if any, are tolerant of modification and which are conserved (i.e. expectedly intolerant to modification), and detailed knowledge of the ways in which the proteins' structure relates to its function. However, in this case the disclosure is limited to a method for hydroxylating 15-para-nitrophenoxycarboxylic acids (pNCA), 12-pNCA, 10-pNCA or 8-pNCA with a polynucleotide encoding a mutant cytochrome P450 monooxygenase of SEQ ID NO:2, wherein the mutant consists of mutations at residue 26, 47, 74, 87, 188 or 354 of SEQ ID NO:2. It would require undue experimentation of the skilled artisan to make the claimed variants and mutants of any P450 monooxygenases and use the claimed variants and mutants of any P450 monooxygenase to produce any subterminally hydroxylated aliphatic carboxylic acids. In view of the great breadth of the claim, amount of experimentation required to make and use the polynucleotides in the claimed method, the lack of guidance, working examples, and unpredictability of the art in predicting function from a polypeptide primary structure, the claimed invention would require undue experimentation. As such, the specification fails to teach one of ordinary

skill how to use the full scope of the polynucleotides encompassed by the claimed method.

While enzyme isolation techniques, recombinant and mutagenesis techniques are known, and it is routine in the art to screen for multiple substitutions or multiple modifications as encompassed by the instant claims, the specific amino acid positions within a protein's sequence where amino acid modifications can be made with a reasonable expectation of success in obtaining the desired activity/utility are limited in any protein and the result of such modifications is unpredictable. In addition, one skilled in the art would expect any tolerance to modification for a given protein to diminish with each further and additional modification, e.g. multiple substitutions.

The specification does not support the broad scope of the claims which encompass a method for the enzymatic production of subterminally hydroxylating aliphatic carboxylic acids using any or all mutants and variants of any P450 monooxygenase, because the specification does not establish: (A) regions of the substrate binding region of any P450 monooxygenase which may be modified without affecting P450 monooxygenase activity or having an altered substrate profile; (B) the general tolerance of P450 monooxygenase to modification and extent of such tolerance; (C) a rational and predictable scheme for modifying any amino acid residue with an expectation of obtaining the desired biological function; (D) aliphatic carboxylic acids which are subterminally hydroxylated with any P450 monooxygenases; (E) a rational and predictable scheme for selecting aliphatic carboxylic acids with an expectation of obtaining a subterminally hydroxylated aliphatic carboxylic acids by incubating said

substrates with any P450 monooxygenase; and (F) the specification provides insufficient guidance as to which of the essentially infinite possible choices is likely to be successful.

Thus, applicants have not provided sufficient guidance to enable one of ordinary skill in the art to make and use the claimed invention in a manner reasonably correlated with the scope of the claims broadly including a method for the production of subterminally hydroxylated aliphatic carboxylic acids using any or all variants and mutants of any P450 monooxygenase. The scope of the claims must bear a reasonable correlation with the scope of enablement (*In re Fisher*, 166 USPQ 19 24 (CCPA 1970)). Without sufficient guidance, determination of mutants and variants of any P450 monooxygenase having the desired biological characteristics recited in the claim is unpredictable and the experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue. See *In re Wands* 858 F.2d 731, 8 USPQ2nd 1400 (Fed. Cir, 1988).

In response to the previous Office Action, applicants have traversed the above rejection.

Applicants argue that the claims are fully enabled since claim 12 has been amended to recite "a nucleic acid sequence encoding a monooxygenase which is derived from *Bacillus megaterium* cytochrome P450 monooxygenase BM-3 with an amino acid sequence comprising SEQ ID NO:1 containing a functional mutation at positions 87 and 188 and optionally at positions 26, 47, 72, 74 and 354. Examiner respectfully disagrees. Applicants' argument on page 9 of the Remarks, that the phrase

"derived *from B. megaterium*" is "not intended to refer to the fact that the sequence is limited to being physically isolated from *B. megaterium"*, is in a way in agreement with Examiner's interpretation that the phrase encompasses any recombinants, variants and mutants of monooxygenase isolated from any other source and labeled as a monooxygenase "derived from *Bacillus megaterium"* in addition to any recombinants, variants and mutants of the monooxygenase having the amino acid sequence of SEQ ID NO:2. Therefore, the claims are not only drawn to mutants of SEQ ID NO:2 comprising the recited substitutions, but any mutants of any monooxygenase "derived" from SEQ ID NO:2 or "derived" from any monooxygenases isolated from any or all source, including any or all mutants, variants and recombinants thereof, comprising the recited substitutions. As discussed above, predictability of which changes can be tolerated in a protein's amino acid sequence and obtain the desired activity requires a specific knowledge of and guidance with regard to which specific amino acids in the protein's sequence, can be modified such that the modified polypeptide continues to have said claimed activity. It is this specific guidance that applicants do not provide. Without specific guidance, those skilled in the art will be subjected to undue experimentation of making and testing each of the enormously large number of mutants that results from such experimentation. While the art may teach in general the structure of P450 monooxygenase, conserved amino acid sequences, and etc, such teachings will not reduce the burden of undue experimentation on those of ordinary skill in the art.

Hence the rejection is maintained.

None of the claims are allowable.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Yong Pak whose telephone number is 571-272-0935. The examiner can normally be reached 6:30 A.M. to 5:00 P.M. Monday through Thursday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ponnathapu Achutamurthy can be reached on 571-272-0928. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 571-272-1600.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

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Primary Patent Examiner 1652

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Short communication

A P450 BM-3 mutant hydroxylates alkanes, cycloalkanes, arenes and heteroarenes

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Abstract

P450 monooxygenases from microorganisms, similar to those of eukaryotic mitochondria, display a rather narrow substrate specificity. For native P450 BM-3, no other substrates than fatty acids or an indolyl-fatty acid derivative have been reported (Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst. Chem. Eur. J. 6 (9), 1531–1536). Engineering the substrate specificity of *Bacillus megaterium* cytochrome P-450 BM3: hydroxylation of alkyl trimethylammonium compounds. Biochem. J. 327, 537–544). We thus were quite surprised to observe, in the course of our investigations on the rational evolution of this enzyme towards mutants, capable of hydroxylating shorter-chain fatty acids, that a triple mutant P450 BM-3 (Phe87Val, Leu188Gln, Ala74Gly, BM3 mutant) could efficiently hydroxylate indole, leading to the formation of indigo and indirubin (Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst. Chem. Eur. J. 6 (9), 1531–1536). Indole is not oxidized by the wild-type enzyme; it lacks the carboxylate group by which the proper fatty acid substrates are supposed to be bound at the active site of the native enzyme, via hydrogen bonds to the charged amino acid residues Arg47 and Tyr51. Our attempts to predict the putative binding mode of indole to P450 BM-3 or the triple mutant by molecular dynamics simulations did not provide any useful clue. Encouraged by the unexpected activity of the triple mutant towards indole, we investigated in a preliminary, but systematic manner several alkanes, alicyclic, aromatic, and heterocyclic compounds, all of which are unaffected by the native enzyme, for their potential as substrates. We here report that this triple mutant indeed is capable to hydroxylate a respectable range of other substrates, all of which bear little or no resemblance to the fatty acid substrates of the native enzyme. © 2001 Elsevier Science B.V. All rights reserved.

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Keywords: P450 BM-3; Alkane hydroxylation; Cycloalkane hydroxylation; Arene hydroxylation; Heteroarene hydroxylation

1. Introduction

CYP102 is a self-sufficient P450 monooxygenase from *Bacillus megaterium* (P450 BM-3) in which the catalytic heme domain and the FAD-containing reductase domain are naturally fused (Li and Poulos, 1997). It has been functionally expressed in *Escherichia coli* and is conveniently prepared and purified in gram quantities (Schwaneberg et al., 1999). Its known function is subterminal, i.e. $(\omega-n)$ hydroxylation of saturated and epoxidation of unsaturated medium- and long-chain fatty acids (Boddupalli et al., 1990; Guengerich, 1991) and its specific activity in this reaction is about 1000-fold higher than eukaryotic P450 monooxygenases of a similar specificity (e.g. CYP52A3 and CYP52A4 from *Candida maltosa*, Scheller et al., 1996). Moreover, *B. megaterium* P450 BM-3 is water-soluble and thus, other than most other members of the P450 family which are multi-protein complexes attached to membranes, can be incorporated in a bioreactor using a zinc-mediator complex instead of expensive NADPH as an external electron donor (Schwaneberg et al., 2000). As the X-ray structure of P450 BM-3 has been resolved at 2.0 Å resolution (Ravichandran et al., 1993), a number of reports have appeared on the putative substrate binding site of this enzyme and have been corroborated by site-directed mutagenesis.

2. Materials and methods

2.1. Enzyme

The P450 BM-3 (Phe78Val, Leu188Gln, Ala74Gly) mutant was obtained as described before (Li et al., 2000).

2.2. NADPH-assay

For the transformations, a 60 mM substrate solution in acetone (50 µl), 50 mM K_xPO_4 buffer (pH 7.5, 9.1 ml), and 2.9 nM (equivalent to 0.05 mg P450, as determined by the carbon-monoxide binding procedure) wild-type or BM-3 mutant enzyme (Li et al., 2000) were combined. For the data reported in Table 1, the mixture was pre-incubated for 5 min, and transformation was started by adding 5 mM aqueous NADPH (300 µl). NADPH consumption was monitored by measuring absorbance at 340 nm. When it had dropped to the blank value, NADPH addition

Table 1
NADPH consumption was measured at 340 nm[a]

Substrates		Reaction rate wild type[b]	Reaction rate mutant[b]
Alkanes, alkanoic acids	Dodecanoic acid	560	788
	Octanoic acid	2.4	35
	Octane	2.5	1760
Alicyclic compounds	α-Ionone	0.8	155
	β-Ionone	1.1	670
Aromatic compounds	Naphthalene	0.8	147
	Anthracene	1.1	45
Heterocyclic compounds	Quinoline	0.9	49
	2-Methylquinoline	1.2	63
	6-Methylquinoline	0.8	58
	8-Methylquinoline	0.9	302

[a] Reaction rates were calculated using an extinction coefficient of NADPH; $\varepsilon = 6.22\ mM^{-1}$.
[b] Reaction rates are given in nmol substrate/nmol P450/min.

was repeated twice (300 μl, 5 mM solution). After complete NADPH consumption, diethyl ether or dichloromethane was added (5 ml), and extraction repeated twice with 5 ml solvent each. The organic phases were combined, dried with $MgSO_4$, and analyzed by thin layer chromatography (TLC, Lutz-Wahl et al., 1998).

2.3. GC-analysis/NMR-analysis

For gas chromatography (GC) and non-magnetic resonance (NMR) analysis, the reaction conditions described above were scaled-up to 10 ml, and NADPH was added in three portions of 100 μl each. GC analyses, Carlo Erba HRGC 4160; on-column injection; column 30 m × 0.25 mm, DB5 (0.25 μm); FID detection. ^{1}H-NMR, Bruker ARX 500, 5 mm dual probe head, $CDCl_3$ solution, 298 K.

3. Results and discussion

The data in Table 1 show indeed that medium-chain alkanes as well as some alicyclic, aromatic, and heterocyclic compounds are good substrates for the BM-3 mutant though not for the wild-type enzyme. Monitoring NADPH consumption is a standard procedure to test for the substrate specificity of P450 monooxygenases (Boddupalli et al., 1990). Since one cannot differentiate, with this experimental setup, between NADPH consumption by productive processes or by side reactions, which leave the substrate unaffected, it is mandatory to prove both structure and amount of any hydroxylation product formed in the course of the enzymatic reaction. For the four most active transformations listed in Table 1, we have complied with this prerequisite by analyzing the reaction mixture extract, after addition of an internal standard, by capillary GLC (with co-injection of authentic standards and/or GC/MS) and, when required, by ^{1}H-NMR.

3.1. Octane (n-alkanes)

On the GLC evidence, octane is transformed by the mutant enzyme almost quantitatively into 2-, 3-, and 4-octanol (molar ratio 1:2.6:2.2, calculated on the basis of the FID response). The chromatogram of the ether extract shows < 1% residual octane substrate but not even a trace of 1-octanol (all assignments verified by co-injection). The three product peaks in the GLC are identified unequivocally by GC/MS (underivatized, EI mode), with the position of the OH functionality clearly demonstrated by the respective $CH_3(CH_2)_{m,n}CH{=}OH^+$ ions for the two α-fragmentation processes.

3.2. α-Ionone (alicyclic compounds)

Judging from NADPH consumption and TLC analysis, both α- and β-ionone are smoothly oxidized. Detailed GC and GC/MS analyses of the α-ionone hydroxylation extract show 70% conversion, mainly to the two diastereoisomeric 3-hydroxy derivatives (49%) formed by a hydroxylation of the CH_2-group adjacent to the ring double-bond (Lutz-Wahl et al., 1998) and to a product which has been hydroxylated in the butenoyl side chain (17%), besides some other minor hydroxy derivatives (< 5% in all).

3.3. Naphthalene (aromatic hydrocarbons)

The GLC for the non-concentrated extract shows only the peaks for the substrate naphthalene and 1-naphthol (FID response 10.3:89.7). When the solvent is gently blown off, though, and a ^{1}H-NMR spectrum of the residue recorded in $CDCl_3$, a set of signals appears (besides those for naphthalene and 1-naphthol) which can be clearly assigned to 1,2-dihydro-1,2-dihydroxynaphthalene, i.e. the characteristic dienediol intermediate of an arene dioxygenation (Engesser et al., 1989). The molar ratio of 13.5:64.0:22.5, determined for these three structures by integration, corresponds to 13.5:86.5 between unreacted substrate and hydroxylated derivatives, virtually identical with the value determined from the FID response. The dienediol apparently suffers regiospecific H_2O elimination to 1-naphthol in the course of the GLC analysis.

3.4. 8-Methylquinoline (heteroarenes)

The gas chromatogram of the original ether extract shows two principal peaks (FID response 61:39). The major one is identified as the substrate peak by co-injection of authentic material. GC/MS verifies this assignment, and establishes the structure of the second peak as a hydroxy methyl quinoline. In concentrating the original extract for NMR analysis by gently blowing off the solvent, 8-methylquinoline is partially taken off with the ether, and the hydroxy product fortuitously enriched in the residue. A detailed analysis of the ^{1}H-NMR spectrum shows that the three-spin system of the heterocyclic moiety is conserved in the hydroxy product down to the coupling fine structure. While 2-H and 3-H are shifted minutely relative to 8-methylquinoline ($+0.7_0$ and -2.1_5 Hz, respectively, i.e. 0.001_4 and -0.004_3 ppm at 11.74 T), 4-H experiences an extreme downfield shift of 0.399 ppm. This is compatible only with the OH substituent in 5-, i.e. in the peri-position. The larger $^3J_{ortho}$ coupling constant (7.6 Hz) for the two remaining protons in the carbocyclic moiety constitutes additional proof for the 5-hydroxy structure. There is evidence from the NMR spectrum also of isomeric substitution products, though in very minor quantities ($< 5\%$ each).

A surprisingly wide substrate specificity, quite contrary to that of the native enzyme, thus has been demonstrated for the triple BM-3 mutant towards aliphatic, alicyclic, and aromatic hydrocarbons as well as towards aza-heteroarenes, though with a strong structural dependence for each specific substrate. Naphthalene, for instance, is hydroxylated, with high efficiency and regioselectivity, to 1-naphthol. Anthracene, on the other hand, is virtually not accepted, and neither is pyridine, 9H-carbazol, or quinoline while 8-methylquinoline is again transformed efficiently.

Surprisingly, n-octane is oxidized at a similar rate as n-dodecanoic acid, a preferred substrate of both the native enzyme and the triple mutant, and about 20-fold faster than octanoic acid. It remains to be seen how n-octane, which lacks any functional group for specific substrate binding, is efficiently oxidized in an (ω–n) fashion which closely parallels the (ω–n) oxidation of fatty acids by native P450 BM-3. The enantioselectivity, in contrast, appears severely impeded, while the native enzyme produces exclusively (ω–n)-hydroxy fatty acids with *R* configuration, alkane hydroxylation by the BM-3 mutant, from our preliminary evidence, gives an *R*/*S* mixture of (ω–n) hydroxyalkanes, although with at least a 2:1 preference in favor of the *R* enantiomers. Incidentally, this new BM-3 mutant was evolved expressly for the oxidation of shorter-chain fatty acids; it oxidizes octanoic acid in (ω–n) position — a reaction which does not take place with wild-type P450 BM-3 (Shao and Arnold, 1996).

For the alicyclic, aromatic, and heterocyclic compounds tested so far, oxidation by the BM-3 mutant is not fully regioselective, though often one regioisomer is formed preferentially, e.g. 1-naphtol from naphtalene or 5-hydroxy-8-methylquinoline from 8-methylquinoline. A computer model of the P450 BM-3 mutant shows all three mutations, which were evolved in a random procedure using saturated mutagenesis (Lentz et al., 2001) to be in or close to the substrate binding pocket. The model gives no indication, though, how these mutations actually influence the unusual catalytic behavior of our BM-3 mutant. This aspect is now being investigated by QSAR and Fe-NMR studies.

The triple BM-3 mutant, obtained by serendipity, may not be the only example of P450 BM-3 mutants with an unusual specificity in substrate oxidation. In view of the potential of P450 monooxygenases for the regio- and stereoselective hydroxylation of non-activated carbon–hydrogen bonds and of the unique advantages of a recombinant P450 BM-3 system we are presently investigating how this enzyme, and mutants thereof, can be exploited in the chemical synthesis of various organic chemicals.

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References

Boddupalli, S.S., Eastabrook, R.W., Peterson, J.A., 1990. Fatty acid monooxygenation by cytochrome P450BM-3. J. Biol. Chem. 265, 4233–4239.

Engesser, K.H., Strubel, V., Christoglou, K., Fischer, P., Rast, H.G., 1989. Dioxygenolytic cleavage of aryl ether bonds: 1,10-dihydro-1,10-dihydroxyfluoren-9-one, a novel arene dihydrodiol as evidence for angular dioxygenation of dibenzofuran. FEMS Microbiol. Lett. 65, 205–210.

Guengerich, F.G., 1991. Reactions and significance of cytochrome P-450 enzymes. J. Biol. Chem. 266, 10019–10022.

Lentz, O., Li, Q.-S., Schwaneberg, U., Lutz-Wahl, S., Fischer P., Schmid, R.D., 2001. Modification of the fatty acid specificity of cytochrome P450 BM-3 from *Bacillus megaterium* by directed evolution: a validated assay. J. Mol. Catal. (B), in press.

Li, H., Poulos, T.L., 1997. The structure of the cytochrome P450BM-3 haem domain complexed with the fatty acid substrate, palmitolic acid. Nat. Struct. Biol. 4, 140–146.

Li, Q.S., Schwaneberg, U., Fischer, P., Schmid, R.D., 2000. Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst. Chem. Eur. J. 6 (9), 1531–1536.

Lutz-Wahl, S., Fischer, P., Schmidt-Dannert, C., Wohlleben, W., Hauer, B., Schmid, R.D., 1998. Stereo- and regioselective hydroxylation of α-ionone by Streptomyces strains. Appl. Environ. Microbiol. 64, 3878–3881.

Ravichandran, K.G., Boddupalli, S.S., Haseman, C.A., Peterson, J.A., Deisenhofer, J., 1993. Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450s. Science 261, 731–736.

Scheller, U., Zimmer, T., Kargel, E., Schunck, W.-H., 1996. Characterization of the n-alkane and fatty acid hydroxylating cytochrome P450 forms 52A3 and 52A4. Arch. Biochem. Biophys. 328 (2), 245–254.

Schwaneberg, U., Sprauer, A., Schmidt-Dannert, C., Schmid, R.D., 1999. P450 monooxygenase in biotechnology. I. Single step, large-scale purification method for cytochrome P450BM-3 by anion-exchange chromatography. J. Chromatogr. A 848, 149–159.

Schwaneberg, U., Appel, D., Schmitt, J., Schmid, R.D., 2000. P450 in biotechnology: zinc driven *w*-hydroxylation of *p*-nitrophenoxydodecanoic acid using P450 BM-3 f87A as a catalyst. J. Biotechnol. 84 (3), 249–257.

Shao, Z., Arnold, F.H., 1996. Engineering new functions and altering existing functions. Curr. Opin. Struct. Biol. 4, 513–518.